Evidence for α-Helices in the Large Intracellular Domain Mediating Modulation of the α1-Glycine Receptor by Ethanol and Gβγ

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ABSTRACT

The α1-subunit containing glycine receptors (GlyRs) is potentiated by ethanol, in part, by intracellular Gβγ actions. Previous studies have suggested that molecular requirements in the large intracellular domain are involved; however, the lack of structural data about this region has made it difficult to describe a detailed mechanism. Using circular dichroism and molecular modeling, we generated a full model of the α1-GlyR, which includes the large intracellular domain and provides new information on structural requirements for allosteric modulation by ethanol and Gβγ. The data strongly support the existence of α-helical conformation in the regions near transmembrane (TM)-3 and TM4 of the large intracellular domain. The secondary structure in the N-terminal region of the large intracellular domain near TM3 appeared critical for ethanol action, and this was tested using the homologous domain of α subunit of the GABA_{A} receptor predicted to have little helical conformation. This region of α2 was able to bind Gβγ and form a functional channel when combined with α1-GlyR, but it was not sensitive to ethanol. Mutations in the N- and C-terminal regions introduced to replace corresponding amino acids of the α1-GlyR sequence restored the ability to be modulated by ethanol and Gβγ. Recovery of the sensitivity to ethanol was associated with the existence of a helical conformation similar to α1-GlyR, thus being an essential secondary structural requirement for GlyR modulation by ethanol and G protein.

Introduction

Glycine receptors (GlyRs) are members of the ligand-gated ion channel receptor (LGIC) superfamily, which includes the Cys-loop family composed of the inhibitory GlyRs and its close relatives, the GABA_{A} receptors (GABA_{A}R), the excitatory nicotinic acetylcholine receptors, and serotonin type 3 receptors (Lester et al., 2004; Lynch, 2004). The α1-subunit containing GlyRs are found mainly in the spinal cord and brainstem neurons, where they participate in motor control, coordination of spinal reflexes, respiratory rhythms, and neuronal development (Legendre, 2001; Lynch, 2004). GABA_{A}R, on the other hand, are found in synaptic and nonsynaptic areas in upper brain regions (Cherubini and Conti, 2001).

GlyRs are pentameric receptors composed of α1–4- and β-subunits, which can assemble to form homeric or heteromic channels. Each subunit is composed of four hydrophobic transmembrane domains (TM1–4), where TM2 contributes to the ion channel formation. The large extracellular aminoterminal domain provides the neurotransmitter binding site (Speranskiy et al., 2007; Pless and Lynch, 2009). Finally, the large intracellular loop domain (LIL) between TM3 and TM4 is important for more recently found regulation of channel activity (Deeb et al., 2007; Carland et al., 2009; O’Toole and Jenkins, 2011; Castro et al., 2012) and is the most variable region of these receptor subunits. Additionally, it presents consensus sites for phosphorylation, modulation by intracellular mechanisms, and interaction with cytosolic and cytoskeletal proteins (Moss and Smart, 2001; Sine and Engel, 2006).

Although ethanol is one of the most frequently used drugs of abuse, the mechanisms of action in the central nervous system are not fully elucidated; however, it is recognized that GlyRs are important molecular targets (Aguayo and Pancetti, 1994; Harris, 1999; Ye et al., 2001). A number of studies have been directed at elucidating the site and mechanism of ethanol action in the GlyRs. To date, the effects of ethanol have been determined to be dependent on sites in 1) TM regions with participation of residues A254, S267, and S288 in the α1 sequence (TM2–TM3) (Crawford et al., 2007); 2) extracellular

ABBREVIATIONS: BAPTA, 2-[2-[2-(bis(carboxyatomethyl)amino)phenoxy]ethoxy]-N-(2-oxoethyl)anilinoacetate; CT, C-terminal; GABA_{A}R, GABA_{A} receptor; CD, circular dichroism; GluCl, glutamate-gated chloride channel; GlyR, glycine receptor; GST, glutathione-S-transferase; IR, intermediate or central region; LGIC, ligand-gated ion channel receptor; LIL, large intracellular loop domain; NT, N-terminal; TM, transmembrane domain; UniProt, Universal Protein Resource.
domains involving A52 (Perkins et al., 2008); and 3) the large intracellular loop domain of GlyRs (Harris et al., 2008; Yevenes et al., 2008). For the last of these, the a1-subunit of the GlyR is modulated by Gβγ through two motifs rich in basic residues (316RFKRR326 and 386KK396) in the LIL (Yevenes et al., 2006), and this protein-protein interaction appears to be an important regulator, even though it is indirect, for ethanol-induced potentiation (Yevenes et al., 2008). A recent study using a chimeric construct of a1-a2-GlyR showed that in addition to these motifs, three residues outside the LIL—A52, S25, and G296—are critical for ethanol modulation in the a1-subunit. Specifically, these mutations caused the loss of ethanol potentiation and suggested that these residues are important for agonist induced conformational changes before channel opening (Yevenes et al., 2010).

Despite the importance of the ethanol effect in GlyRs, there are still limited structural data on the events leading to the potentiation of glycineric currents by ethanol. Recently, some binding sites for ethanol in TMs have been studied using structures in crystallized channels, such as glutamate-gated chloride channel (GlCiU) (Hibbs and Gouaux, 2011) or prokaryotic Glocobacter ligand-gated ion channel (GLCi/Veroxin chrysanthemi) ligand-gated ion channel (ELIC) (Bocquet et al., 2009; Howard et al., 2011; Corringer et al., 2012; Murial et al., 2012; Olsen et al., 2014). However, these protein structures lack the LIL, hindering a more detailed analysis of the events that lead to potentiation of GlyR by low concentrations (<100 mM) of ethanol through activation of Gβγ. Therefore, using secondary structure prediction, circular dichroism (CD), and chimeric receptors, we examined the relationship between the secondary structure of the LIL of a1-GlyR subunits and its sensitivity to ethanol. The results support the existence of a helical conformation in the N-terminal region of the LIL in a1-GlyR subunits that is associated with the ability of ethanol to potentiate GlyR through activation of Gβγ. This α-helix corresponds to a novel, necessary structural requirement for modulation by ethanol. Additionally, these data provide structural information about the LIL and its association with mechanisms of channel modulation, which is relevant because lack of the LIL in prokaryotic LGICs hampers potentiation by low concentrations of ethanol (Howard et al., 2011; Sauguet et al., 2013).

**Materials and Methods**

**Secondary Structure Predictions.** All predictions were made using the coding sequences of a1 (UniProt Protein Resource (UniProt) P07727), a2 (UniProt: P22771), a3-GlyR (UniProt: P24524), and y2-GABA AR (UniProt: P18508) subunits from Rattus norvegicus. Three programs that incorporate different parameters in their prediction methods were used: PSIPRED (Department of Computer Science, Bioinformatics Group, University College of London, London, UK) (Buchan et al., 2013), JUFO (Meiler Lab, Vanderbilt University, Nashville, TN) (Leman et al., 2013), and Jpred3 (Cole et al., 2008). After all secondary structure predictions were finished with each of the programs used, we combined them to create a consensus secondary structure where each structural element (helix, β-sheet, random) was confirmed by at least two of the prediction servers used.

**Protein Expression of the LILs.** DNA constructs encoding the complete LIL of the a1-GlyR subunit (amino acids 309–393), three truncated intracellular loops corresponding to the N-terminal (NT; amino acids 309–325), an intermediate or central region (IR; amino acids 326–381), and C-terminal (CT; amino acids 356–392), and the full sequence of the LIL of the y2-GABA AR subunit (amino acids 315–411) were cloned into the pGEX-SX-3 vector (GE Healthcare Life Sciences, Pittsburgh, PA). Glutathione-S-transferase (GST) fusion proteins were expressed in Escherichia coli BL21 cells and harvested by centrifugation, followed by resuspension in lysis buffer (1× phosphate buffer, 1% Triton X-100, and protease inhibitor mixture set II) (Calbiochem, San Diego, CA), treatment with lysozyme (0.1 mg/ml, 30 minutes, 0°C), and sonication on ice collecting the supernatant. Expressed a1-GlyR and truncated proteins were purified by chromatography in a fast protein liquid chromatography system with a GSTrap FF column (GE Healthcare Life Sciences), and y2-protein was purified with a glutathione resin (Novagen; Merck Biosciences, Whitehouse Station, NJ), all according to the manufacturer’s protocol, using 50 mM Tris-Cl and 10 mM reduced glutathione (pH 8.0) for elution. Finally, proteins were dialyzed with 10 mM sodium phosphate buffer, pH 7.0.

**Circular Dichroism of GST-Fusion Proteins.** Measurements of CD were performed on a Jasco-J815 spectropolarimeter (Jasco Products Company, Oklahoma City, OK) in a 0.1-cm path-length quartz cell. Direct CD spectra for the LIL of a1-GlyR subunits, truncated proteins, and the LIL of y2-GABA AR were recorded in the spectral range of 200–250 nm at 0.5-nm intervals. Ten individual scans at 20°C were taken and averaged. All spectra were baseline-corrected by subtracting buffer runs and GST contribution. Protein concentrations were determined by measuring the absorbance at 280 nm, with an extinction coefficient calculated from the amino acid sequences using ProtParam (http://web.expasy.org/protparam/). The content analyses of secondary structures were performed from the CD spectra using the K2D3 server (Louis-Jeune et al., 2012).

**GST Pull-Down.** GST fusion proteins containing the LIL of the a1-GlyR subunit and the y2-subunit of the GABA AR were expressed in the E. coli BL21 strain by induction with isopropyl β-D-thiogalactopyranoside as described previously (Yevenes et al., 2006). Briefly, the expressed proteins were purified using a glutathione resin (Novagen). Normalized amounts of GST fusion proteins attached to glutathione resins were incubated with Gβγ proteins (Calbiochem). After washing, the presence of Gβ was detected by SDS-PAGE (12% acrylamide). For Western blot analysis, the proteins were transferred to a nitrocellulose membrane, followed by immunodetection with an anti-Gβ antibody (1:200; Santa Cruz Biotechnology, Inc., Dallas, TX). Signal detection was measured using a chemiluminescence kit (PerkinElmer Life Sciences, Waltham, MA) and quantified by densitometry.

**Molecular Modeling.** Models of the α1-GlyR, y2-GABA AR, and chimera a1-LIL y2-subunits were constructed by homology using coordinates from the Caenorhabditis elegans GluCl at 3.3 Å resolution (Hibbs and Gouaux, 2011) (PDB ID 3RIF) using the software Modeller 9v10 (Sverar, 2006). Because of the lack of sequence identity with a protein having a defined structure, the large intracellular domains of a1- and y2-subunits were predicted by ab initio technique using QUARK (Denver, CO) (Xu and Zhang, 2010). In the next step, large intracellular domains were combined with their respective subunit models using Modeller to generate complete models of α1 and y2. Finally, the models were relaxed by energy minimization using a conjugate gradient protocol in the software MacroModel (version 9.9, 2012, Schrödinger, LLC, New York, NY). All images presented were created with PyMOL (Schrödinger, LLC).

**Construction of Chimera a1-LIL-y2.** The cDNA constructs encoding the rat glycine receptor α1-subunit subcloned in a pCI vector (Promega Corporation, Madison, WI) have been described previously (Yevenes et al., 2010). For the construction of chimera a1-LIL-y2, an XbaI site was added in the TM3 domain of the α1-GlyR to replace the large intracellular domain up to the C terminus with the homologous region of the y2-GABA AR subunit. Mutations were inserted in the TM4 and C-terminal to correct the sequence and convert it into an α1-GlyR using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All the constructions were confirmed by full sequencing. The glycine and GABA AR amino acids were numbered according to their position in the mature protein sequence.

**Cell Culture and Transfection.** HEK293 cells were cultured using standard methods. HEK293 cells were transfected using βXect (Clontech Laboratories, Inc., Mountain View, CA) with 1 μg of DNA for each plasmid studied per well (35 mm). Expression of green fluorescent
protein was used as a marker of positively transfected cells with a cotransfection ratio 2:1 of plasmids. Recordings were made after 18–24 hours.

**Electrophysiology.** Whole-cell recordings were performed as previously described (Castro et al., 2012). A holding potential of ~60 mV was used. Patch electrodes were filled with (in mM): 140 CsCl, 10 BAPTA (2-[2-[2-[bis(carboxymethyl)amino]ethyl]amino]ethoxy)N-(2-oxethyl)amino) acetate), 10 HEPEs, pH 7.4, 4 MgCl2, 2 ATP, and 0.5 GTP. The external solution contained (in mM): 150 NaCl, 5.4 KCl, 2.0 CaCl2, 1.0 MgCl2, 10 HEPEs (pH 7.4), and 10 glucose. The resulting glycine activated current was measured at a range of concentrations (10–3000 μM). With this, and using a logistic model, we built a concentration-response curve to obtain the values of EC50 and Hill coefficient. The amplitude of the maximal current was not analyzed because of the variability in the number of receptors expressed in each cell after transient transfection. For G protein activation experiments, GTP in the internal solution was replaced by GTPγS (0.5 mM; Sigma-Aldrich, St. Louis, MO). Normalized values were obtained by dividing the current amplitude obtained with a time of 15 minutes of GTPγS dialysis by the current at the minute 1. The sensitivity of recombinant and chimeric receptors to ethanol were assayed using a pulse of glycine (EC50) coupled with 100 μM ethanol (Sigma-Aldrich) to each receptor studied, without any preapplication. Although ethanol potentiates GlyRs starting at a concentration of 10 μM (Aguayo and Pancetti, 1994; Aguayo et al., 1996), in this study, we used 100 μM to compare the results with other previous studies and to make the statistical analysis more robust. In all the experiments, a brief pulse of 1 mM glycine was performed at the end of the recording period to test that the glycine concentration corresponded to the actual EC50 in each single experiment. Cells having responses < EC50 or > EC50 were discarded. Strychnine (1 μM) blocked all the currents elicited by wild-type and chimeric glycine receptors.

**Data Analysis.** Statistical analyses were performed using analysis of variance and are expressed as the arithmetic mean ± S.E.M.; values of P < 0.05 were considered statistically significant. For all the statistical analysis and plots, the Origin 9.0 (MicroCal, Northampton, MA) software was used.

## Results

**Analysis of the Secondary Structure of the α1-GlyR with Molecular Modeling and CD Spectroscopy.** In the absence of available structures for the GlyR LIL or other members of LGICs, we performed secondary structure predictions from the sequence of α1-subunits, focused primarily on the LIL (Supplemental Fig. 1A). Analysis of the LIL of α1-, α2-, and α3-subunits showed two regions with a high probability to form a α-helix structure near the TM3 (N terminus) and TM4 (C terminus). The N-terminal helix includes the cluster of basic amino acids 316RFKRR320 described previously as essential for modulation by ethanol and Gβγ (Yevenes et al., 2008). On the other hand, the amino acids 381KKKK385 were found to be adjacent, but not encompassed, in the helical structure predicted in the C-terminal portion of the LIL.

With the aim of constructing a full model of the α1-GlyR subunit containing the LIL, we predicted the LIL by ab initio using Quark (Xu and Zhang, 2012) and combined it with the structure of the GluCl (Hibbs and Gouaux, 2011). The model of α1 confirmed the secondary structure predictions, with the presence of an α-helix structure in the LIL of α1 (Fig. 1A). It is interesting that the orientation of the LIL appears flexible and slight variations are expected, especially in the angle between the LIL and the TM3–TM4 domains. It is possible that this flexibility is due to the presence of random regions connecting the α-helices predicted near the TM3, and this conformation might allow the formation of a channel pore and to facilitate its interactions with regulatory cytoplasmic proteins (Fig. 1B).

To confirm the presence of the helical conformation predicted by in silico analysis of the α1-GlyR, we examined the secondary structure of the LIL using CD spectroscopy. For this, four GST fusion proteins were constructed consisting of the full LIL of α1 (GlyR-LIL), the N terminus of the LIL, including residues 309–325 (GlyR-N), an intermediate or central region between residues 326–381 (GlyR-IR), and the C-terminal region from residue 356–392 (GlyR-CT) (Fig. 2A). All proteins were analyzed by SDS-PAGE to confirm correct expression and purification steps (Supplemental Fig. 2). The CD spectra (Fig. 2B) for α1 confirmed that its secondary structure presents helical conformations (27%), together with β-sheet (22%), and the remaining exhibiting a random structure, in agreement with the expected flexibility of the LIL (Fig. 2C). Constructs that included only regions near the TM domains 3 and 4 showed an even higher content of helical structure. For example, GlyR-NT exhibited 34% and GlyR-CT 31% of α-helix, supporting the conclusion that this secondary structure is present at the boundaries of the LIL. The central segment of the LIL displayed the greatest proportion of random structure (GlyR-IR, 54%) probably linked to the flexibility required for its interaction with other cytoplasmic proteins. Finally, the percentage of secondary structure calculated from circular dichroism spectra were used to validate the model of the α1 GlyR, and we found a good correlation between our model and CD analysis (32% α-helix in the LIL of the α1 model located in the N- and C-terminal regions).

**Protein Interaction between the LIL of α1-GlyR and γ2-GABAAR Subunits with Gβγ.** The secondary structure prediction analysis showed that, similar to α1, the α2-subunit...
of the GlyR presented a high level of helix content (Supplemental Fig. 1, B and C) that was previously found to be important for the modulation by ethanol and Gβγ. For instance, a previous study showed the ability of the LIL in α1- and α2-GlyR to biochemically interact with Gβγ (Yevenes et al., 2010), and this is supported by a high similarity in their sequence, presenting basic clusters at amino acids 316–320 and 385–386 (Fig. 3A). The chimera between α1 and the LIL of α2 interacted with Gβγ and was significantly potentiated by ethanol (Yevenes et al., 2010), which could be attributed to the similar structure in the LILs. To test whether a new chimera with a prediction of low or no secondary structure content in the corresponding LIL region was still sensitive to ethanol and Gβγ modulation, we constructed a α1-γ2-GABAA,γR chimera. We chose γ2 because, in addition to the low full-sequence similarity (35%) with α1-GlyR, it had a similar LIL size (86/87 amino acids in α1/γ2). Furthermore, the LIL of γ2 showed a mainly random structure calculated from circular dichroism data (80%) with a reduced percentage of helical conformation (6%) (Fig. 2, B and C), also in agreement with our initial prediction (Supplemental Fig. 1D) Moreover, the LIL of γ2 has a basic amino acid cluster near TM3 (residues 328–333) with a lysine at position 401 corresponding to lysine 386 in the α1-GlyR that was described as essential for ethanol potentiation (Fig. 3A). Interestingly, the γ2-subunit was described as a potential target for ethanol effects in the central nervous system with a synaptic distribution (Mihic, 1999; Loh and Ball, 2000; Olsen and Sieghart, 2009).

Using a GST pull-down assay, we found that the LIL of γ2 was able to interact with Gβγ to a lower degree than the α1-GlyR (4% ± 10% versus 90% ± 13%) (Fig. 3, B and C). This indicates that the LIL of γ2-GABAA,γR binds to Gβγ but does not necessarily mean that it is directly associated to the modulation of the receptor. Therefore, functional experiments using a chimeric receptor were required to elucidate this aspect.

**Modulation of Chimeric Receptor α1-LIL-γ2 by G Protein and Ethanol.** We constructed a chimeric α1-LIL-γ2 receptor where the LIL of α1-GlyR was replaced by the corresponding LIL of γ2-GABAA,γR (Fig. 4A). We then examined whether this chimera was modulated by ethanol and Gβγ to confirm the importance of the helical conformation in the LIL for allosteric modulation. Remarkably, this chimera produced a highly normal homomeric receptor, with an EC50 of 56 ± 4 μM and a Hill coefficient (nH) of 1.5 ± 0.1 (compared with an EC50 of 40 ± 1 and an nH of 2.4 ± 0.1 for α1 GlyR), indicating that the receptor was correctly expressed and localized in the cell membrane (Fig. 4B). Experiments using intracellular GTPγS to activate G proteins showed that the chimera was insensitive to modulation by Gβγ, and no significant change in the amplitude of the glycine evoked current was detected after dialysis of GTPγS for 15 minutes (Fig. 4, C and D). Furthermore, ethanol had no significant effect on the activity of the chimeric receptor tested at a concentration of 100 mM (15% ± 4%) (Fig. 4, E and F).

These results suggest that the lack of modulation of the chimera might be due to structural differences between α1-GlyR and α1-LIL-γ2. Therefore, we constructed a predictive model that might account for these differences. Examination of the resulting model revealed important differences in the secondary structures of the LILs. In particular, the helical conformation present in the LIL of α1 was absent in the LIL of γ2 (Supplemental Fig. 3), and this is in agreement with the results previously described in secondary structure predictions of both subunits based on sequence analysis (Supplemental Fig. 1). Thus, this
difference in secondary structure could be implicated in the insensitivity of the chimera to GTP\(\gamma\)S and ethanol. To test this possibility, we mutated these regions to match the sequence found in \(\alpha\)1-GlyR previously reported to be important for allosteric modulation by ethanol.

**Recovery of G Protein and Ethanol Modulation in the Chimera**

\(\alpha\)1-LIL-\(\gamma\)2. To learn about the reason for the insensitivity of the chimeric receptor \(\alpha\)1-LIL-\(\gamma\)2, we performed a sequence analysis of \(\gamma\)2 and found a significant difference in the region near the TM3 domain (Fig. 5A). In particular, the sequence of \(\gamma\)2 shows a proline at position 326 in the LIL, a residue that can prevent the formation of helical structures as described in the \(\alpha\)1-GlyR subunit. Also, the C terminus of \(\gamma\)2-LIL lacked the equivalent lysine 385, recognized as important for modulation by ethanol, and presents an alanine 400 in this position (Castro et al., 2012). Therefore, we mutated the segment near the TM3 (309NRKPS313) in the chimera \(\alpha\)1-LIL-\(\gamma\)2 to an \(\alpha\)1-GlyR sequence (309RQHKE313), together with the mutation A385K (Fig. 5A). The electrophysiologic properties of this mutated chimera denoted as \(\alpha\)1-LIL-\(\gamma\)2DLIL were similar to \(\alpha\)1-GlyR, displaying an EC\(_{50}\) of 6455 M and an \(n_H\) of 2.860.4 (Fig. 5B). Furthermore, the predicted model in silico showed these mutations allowed the formation of a helical structure in the regions near TM3 and -4, similar to that found in the LIL of the \(\alpha\)1-GlyR subunit (Fig. 5C).

Interestingly, this modified chimera was potentiated by the dialysis of GTP\(\gamma\)S with an increase in the current evoked by glycine by 35%65% (Fig. 6A). Also, this receptor was sensitive to ethanol and displayed 42%61% potentiation of the glycine-evoked current in the presence of 100 mM ethanol (Fig. 6, C and D). Taken together, our results confirm a structural requirement for the modulation of the chimera \(\alpha\)1-LIL-\(\gamma\)2, transforming an insensitive receptor into one capable of being potentiated by ethanol through G\(\beta\)\(\gamma\). This change in sensitivity is associated with the recovery of the helical structure in the LIL, specifically in the regions near the transmembrane domains.

**Discussion**

The main aim of the present study was to provide information about the structural requirements of the LIL of GlyRs for ethanol-induced modulation. The present data support the existence of a helical structure in the intracellular domain of the \(\alpha\)1-GlyR that is critical for interaction with G\(\beta\)\(\gamma\) and potentiation by ethanol.

It is well accepted that inhibitory GlyRs are modular proteins formed by the N-terminal extracellular domain that contains the agonist binding site, TM domains forming the pore structure and the large intracellular loop domain connecting the TM3 and -4 (Thompson et al., 2010). It is now accepted that charged residues in the LIL have roles in ion channel conductance (Deeb et al., 2007; Gee et al., 2007; O’Toole and Jenkins, 2011). For example, mutations of positive amino acids in the C-terminal region of the LIL decreased the conductance of the channel (Hu et al., 2006), ion selectivity (Livesey et al., 2008; Peters et al., 2010), receptor assembly, targeting, and trafficking (Boyd et al., 2002; Melzer et al., 2010) are affected by modifications in the LIL. It was also shown that the LIL is involved in the modulation of GlyR by ethanol, via an interaction...
between basic amino acids with Gβγ dimers (Yevenes et al., 2008). Despite this wide range of modulatory actions, the LIL is not essential for the formation of a functional channel, as evidenced using truncated and chimeric receptors (Perán et al., 2006; Jansen et al., 2008; Goyal et al., 2011).

Because of its high flexibility and complex protein expression, there is no information yet on the atomic structure of LGICs, including the complete LIL (Bocquet et al., 2009; Hilf and Dutzler, 2009; Hibbs and Gouaux, 2011). This lack of information has hindered understanding of the mechanisms involved in the intracellular modulation of these receptors. To circumvent this problem, we performed predictive analysis of the secondary structure together with CD confirming the presence of α-helix structures (27%) in the full-length LIL, particularly near the TM domains (Figs. 2 and 3; Supplemental Fig. 1). Interestingly, this helical content was slightly lower than previously reported for α3-GlyR, suggesting a high degree of structure conservation between α-subunits of GlyR (Breitinger et al., 2009). Also, a potential helical structure in the N-terminal LIL region was predicted in silico in pharmacologic studies of ethanol using small synthetic peptides derived from these regions (Guzman et al., 2009; San Martin et al., 2012). The presence of these helical structures is likely to serve for protein-protein interactions of the type previously proposed for other proteins (Bonacci et al., 2005; Everitt et al., 2009; Chin et al., 2013; Shim et al., 2013; Alexander et al., 2014) and antagonized by small peptides having helical structures.

**Fig. 4.** Characterization of the chimeric receptor α1-LIL-γ2 sensitivity to G protein and ethanol modulations. (A) Schematic representation shows the generation of the chimera α1-LIL-γ2. (B) Glycine concentration-response relationship for the α1-GlyR and the chimera α1-LIL-γ2. (C) Representative glycine-evoked current traces at the beginning (1 minute) and after 15 minutes of intracellular dialysis of the nonhydrolyzable analog GTPγS in HEK cells expressing the chimera α1-LIL-γ2. The concentration of glycine was 15 μM (EC10 – 20). (D) Effects of GTPγS on α1-GlyR and chimera α1-LIL-γ2 after 15 minutes of dialysis in terms of the percentage of potentiation of evoked currents in relation to the initial evoked current. (E) Representative glycine-evoked current traces (15 μM) in the absence and presence of 100 mM ethanol in α1-LIL-γ2. The concentration of glycine was 15 μM (EC10 – 20). (F) Effects of 100 mM ethanol on α1-GlyR and chimera α1-LIL-γ2 in terms of percent of potentiation by 100 mM ethanol of 15 μM glycine-evoked currents. **P < 0.001. Data are mean ± S.E.M. (n = 6).

**Fig. 5.** Characterization of chimeric receptor α1-LIL-γ2 ΔLIL containing homologous replacements of amino acids in regions near TM3 and -4. (A) Mutations in the chimera α1-LIL-γ2 to include the residues present in the α1-GlyR subunit. The α1-LIL-γ2 ΔLIL construct includes the cluster 309–313 RQHKE (blue) and the 385–386 basic cluster KK (red). (B) Glycine concentration-response curves for α1-GlyR, chimera α1-LIL-γ2 and modified chimera α1-LIL-γ2 ΔLIL. (C) Homology model of chimera α1-LIL-γ2 ΔLIL having a recovery of helical structure similar to that observed in the model of the α1-GlyR subunit.
Fig. 6. Modulation of chimeric receptor α1-LIL-γ2 ΔLIL by Gβγ and ethanol. (A) Glycine-evoked current traces at the beginning (1 minute) and after 15 minutes of intracellular dialysis of the nonhydrolyzable analog GTPγS in HEK cells expressing the chimera α1-LIL-γ2 ΔLIL. The concentration of glycine was 15 μM (EC_{50}=20). (B) Effects of GTPγS on chimera α1-LIL-γ2 and its modified version α1-LIL-γ2 ΔLIL after 15 minutes of dialysis as the percentage of potentiation of evoked currents with respect to the initial current amplitude. (C) Glycine-evoked current traces (15 μM) in the absence and presence of 100 mM ethanol recorded in HEK cells expressing the chimera α1-LIL-γ2 ΔLIL. (D) Effects of 100 mM ethanol on chimera α1-LIL-γ2 and its modified version α1-LIL-γ2 ΔLIL shown as percent potentiation by 100 mM ethanol of 15 μM glycine-evoked currents. **P < 0.001. Data are mean ± S.E.M. from at least four cells.

Furthermore, using a chimera made of α1-GlyR and the LIL from γ2-GABAAR, a sequence almost without α-helices (Fig. 2C), we obtained new data that support our conclusion that the presence of this type of conformation in the N-terminal part of the LIL region in the α1-GlyRs is required for ethanol modulation (Fig. 6). Likewise, a similar structure was predicted in the C-terminal part of the LIL region. Thus, this type of chimeric receptor approach adds new information to identify residues that are critical for ethanol actions in TM domains in GlyRs and GABAARs (Mihic et al., 1997). Moreover, another recent study used a chimera between α1-GlyR and δ-GABAAR to identify residues in the extracellular loop 2 of GlyRs that generated a “super ethanol sensitive” conformation (Perkins et al., 2009).

Based on GST pull-down studies, we determined that the LIL of the γ2 subunit was able to bind reasonably well to the Gβγ dimer (Fig. 3B). The ability to bind Gβγ is not directly associated to modulation of receptor activity, for example, the α2-GlyR subunit binds Gβγ, but it is not potentiated by G protein or ethanol (Yevenes et al., 2010). In this sense, the chimera α1-LIL-γ2 was not modulated by G protein and ethanol, suggesting the existence of other structural differences (Fig. 4, C–F). This result was somewhat surprising because a previous study with the chimera α1-α2-GlyR where the LIL of the α1-subunit was replaced by the corresponding one of α2 showed that this receptor was highly sensitive to ethanol modulation indicating a modular receptor function (Yevenes et al., 2010). Interestingly, the chimera α1-LIL-γ2 presented no large differences in properties to the α1-GlyR (EC_{50}=56 ± 4 μM versus EC_{50}=40 ± 1 μM) (Fig. 4B), which could explain the insensitivity to ethanol and G protein because of reduced gating toward an open state. Additional analysis showed that mutations in the chimera α1-LIL-γ2 to corresponding residues found in α1-GlyR resulted in a phenotype similar to that of α1-GlyR (EC_{50}=49 ± 1 μM), including modulation by GTPγS (35% ± 5%) and ethanol (41% ± 2%) (Figs. 5 and 6). Thus, these sites mutated in the LIL of γ2 produced an α-helix next to the TM regions that is important for the phenotype. These new results illustrate the requirement of several properties for ethanol modulation in the α1-GlyR subunit, which are also present in the chimera α1-LIL-γ2 ΔLIL and correspond to the presence of a basic cluster in the LIL N-terminal region, two lysines in the LIL C-terminal region (Yevenes et al., 2008), and the presence of helical structures near the TM3 and TM4 regions. Additionally, the present results validate a complete model of the α1-GlyR that demonstrates a high degree of helical structure in the LIL that might be necessary for interaction with helical structures in the “hot spot” in Gβγ (Figs. 2 and 3) (Davis et al., 2005; Smrcka, 2008).

In conclusion, the present study suggests that the amino acids located in the large intracellular loop near TM3 and TM4 induce a conformation in the LIL that allows the receptor to be modulated by ethanol and Gβγ. This conformation comprises an α-helix structure and indicates that this feature is critical for ethanol potentiation via Gβγ.

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Authorship Contributions
Participated in research design: Burgos, Castro, Aguayo.
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Performed data analysis: Burgos, Castro, Aguayo.
Wrote or contributed to the writing of the manuscript: Burgos, Castro, Guzmán, Aguayo.

References


Castronovo V, Murphy M, Gangadharan GE, Orien ML, and Aguayo LG (2012) The basic property of La3+ is important for the potentiation of the α1 glycine receptor by ethanol. J Pharmacol Exp Ther 340:539–549.


